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# STEREOCHEMISTRY OF THE KDO8P SYNTHASE. AN EFFICIENT SYNTHESIS OF THE 3-FLUORO ANALOGUES OF KDO8P

**Amnon Kohen, Ronit Berkovich, Valery Belakhov and Timor Baasov***
Department of Chemistry, Technion - Israel Institute of Technology
Technion City, Haifa 32000, Israel

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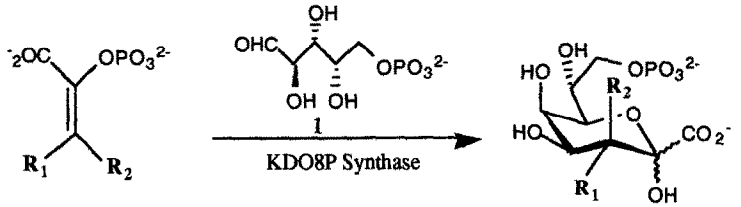
**Abstract:** Direct evidence of the overall stereochemistry of the KDO8P synthase reaction was obtained by using 3-deuterio and 3-fluoro analogues of phosphoenolpyruvate as alternative substrates and by subsequent $^{1}H$ and $^{19}F$ NMR analysis of the enzymatic reaction products. The results reveal the *si* face addition of enzyme-bound phosphoenolpyruvate to the *re* face of the carbonyl of arabinose 5-phosphate.

3-Deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase [EC 4.1.2.16] is a key enzyme that controls the carbon flow in the biosynthetic formation of 3-deoxy-D-*manno*-2-octulosonate (KDO).[1] This unusual eight-carbon sugar is a site-specific constituent of the lipopolysaccharide of most Gram-negative bacteria, and provides the link between lipid A and the growing polysaccharide chain.[2] Since the synthesis and activation of KDO is a vital part of the assembly process of lipopolysaccharides in Gram-negative bacteria, several research groups have recently pursued inhibition of KDO metabolism as a strategy for the development of novel antiinfective agents.[3]




**2**, $R_1 = R_2 = H$
**4**, $R_1 = H$; $R_2 = {}^{2}H$
**5**, $R_1 = {}^{2}H$; $R_2 = H$
**6**, $R_1 = H$; $R_2 = F$
**7**, $R_1 = F$; $R_2 = H$

**3**, $R_1 = R_2 = H$
**8**, $R_1 = H$; $R_2 = {}^{2}H$
**9**, $R_1 = {}^{2}H$; $R_2 = H$
**10**, $R_1 = H$; $R_2 = F$
**11**, $R_1 = F$; $R_2 = H$

KDO8P synthase catalyzes the condensation of D-arabinose 5-phosphate (**1**, A5P) with phosphoenolpyruvate (**2**, PEP) to produce KDO8P (**3**) and inorganic phosphate.[4] The unusual chemistry of the enzyme catalysis and its biological importance coupled with the ambition to design a new mechanism-based KDO8P synthase inhibitor which could in principle provide new selective antibiotic of commercial significance,

prompted us to investigate the mechanism of this enzyme reaction. It has been shown[5] that the reaction proceeds through the C-O bond rather than P-O bond cleavage of PEP. We have recently determined the anomeric specificity for the substrate A5P and demonstrated an ordered sequence of substrate binding (PEP followed by A5P) and product release (Pi prior to KDO8P).[6] After extending our studies on KDO8P synthase mechanism, we are able to describe here for the first time the steric course of the enzyme catalyzed reaction.

The side of attack at the carbonyl of A5P was already evident as a *re* face from the known *manno* configuration of KDO8P. However, the side of PEP addition was still compatible for either the *si* or *re* face of the plane of enzyme-bound PEP. In order to solve the stereochemistry of PEP addition, stereospecifically labeled samples of PEP at C-3 (structures **4-7**) were prepared and used as alternative substrates for the synthase. The enzymatic reaction products were isolated and the exact configuration at the C-3 center was unambiguously assigned by $^{1}H$ and $^{19}F$ NMR.

**Table 1.** $^{1}H$ NMR Chemical shifts and coupling constants for H-3 protons of KDO8P (**3**), (3S)-[3-$^{2}H$]KDO8P (**8**) and (3R)-[3-$^{2}H$]KDO8P (**9**).[a]

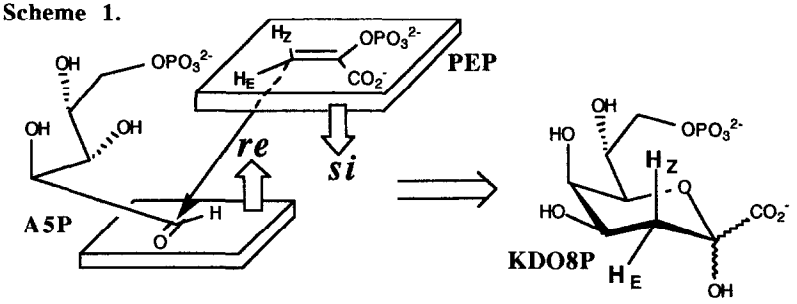
| Compound | α-pyranose | β-pyranose | α-furanose[b] | β-furanose[b] |
|---|---|---|---|---|
| | δ(*J*) | δ(*J*) | δ(*J*) | δ(*J*) |
| **3** | $H_a$: 1.76 (13.4, 12.9) | $H_a$: 1.58 (13.4, 12.8) | H': 1.89 (14.2, 3.16) | H': 2.12 (13.3, 7.0) |
| | $H_e$: 1.70 (13.4, 5.7) | $H_e$: 2.15 (13.4, 5.6) | H": 2.40 (14.2, 7.2) | H": 2.20 (13.4, 7.0) |
| **8** | $H_e$: 1.69 (5.0) | $H_e$: 2.12 (4.4) | H": 2.38 (7.2) | H": 2.17 (6.95) |
| **9** | $H_a$: 1.75 (11.8) | $H_a$: 1.59 (11.8) | H': 1.89 (2.6) | H': 2.12 ((7.0) |

[a]Recorded at 400 MHz for the sodium salts in $D_2O$, pD 7.0, referenced to HOD at 4.63 ppm. Values of chemical shifts measured in ppm. Values of coupling constants are given in parentheses and measured in Hz. $H_a$ and $H_e$ refer to the axial and equatorial orientation, respectively, of the H-3 proton in the pyranose anomers. The similar orientation in the furanose anomers are designated by H' and H". [b]The assignments of the furanose resonances to α and β are tentative.

The synthesis of (*Z*)-[3-$^{2}H$]PEP (**4**) proceeded from commercially obtained ethyl bromopyruvate via ethyl (*Z*)-3-deuterio-2-[(dimethoxyphosphinyl)oxy]-propenoate,[7] which was then smoothly deprotected according to the procedure of Bartlett.[8] Incubation of **4** with KDO8P synthase[9] provided [3-$^{2}H$]KDO8P (**8**) which was isolated in pure form by anion-exchange chromatography. $^{1}H$ NMR analysis (Table 1) of this material and its comparison with the spectrum of the unlabeled KDO8P (**3**)[10] indicated that the deuterium label was entirely in the axial position, establishing the (3*S*) configuration in **8**. Furthermore, when similar experiments were done by using various *Z-E* mixtures of [3-$^{2}H$]PEP,[7] containing increased amounts of *E* isomer (**5**) [*Z* to *E* ratio of 85:15, 70:30 and 40:60], a subsequent increase of (3*R*)-[3-$^{2}H$]KDO8P (**9**) was observed. The new stereogenic centers (3*S* in **8** and 3*R* in **9**) were diagnosed by the coupling constants of H-3 protons in the major α-pyranose products (Table 1). This coupling constant in **8** was 5 Hz which is consistent with the gauche ($H\text{-}3_e$, $H\text{-}4_a$) arrangement of H-3 and H-4 protons and thus confirms the 3*S* configuration. However, the coupling constant of 11.8 Hz in **9** is only consistent to the *trans*-diaxial ($H\text{-}3_a$, $H\text{-}4_a$) relationship indicating that the deuterium atom is oriented in an equatorial position. This arrangement of atoms in **9**, *i.e.*,

axial H-3 proton and equatorial deuterium, confirms the *R* configuration at C-3. Thus the results indicate that, the KDO8P synthase catalyzed reaction is predominantly stereospecific with respect to C-3 of PEP. Furthermore, since the (*Z*)-[3-$^2$H]PEP (**4**) gave rise to (3*S*)-[3-$^2$H]KDO8P (**8**) and the *E* isomer (**5**) produced the opposite 3*R* diastereomer (**9**) of [3-$^2$H]KDO8P, the present observation dictates that the attachment of C-3 of PEP to the carbonyl of A5P must proceed from the *si* face of the enzyme-bound PEP as illustrated in Scheme 1.

**Scheme 1.**




Further support for the above results was obtained from the experiments carried out with *Z* and *E* isomers of phosphoenol-3-fluoropyruvate ([3-F]PEP) as alternative substrates of the synthase. Incubation[9] of (*Z*)-[3-F]PEP (**6**, >95% stereochemical purity)[11] with the synthase and monitoring of the reaction progress by $^{19}$F NMR, clearly showed the gradual consumption of the substrate at -141.7 ppm and the subsequent appearance of four signals at -203.8, -206.7, -209.8 and -214.2 ppm. These same four signals were recorded for the clean product (Fig. 2a) obtained after purification by ion-exchange chromatography. When a similar experiment was carried out with (*E*)-[3-F]PEP (**7**, >95% stereochemical purity),[11] the reaction was much faster and the appearance of three new signals was noted. Accordingly, the reaction with *Z*-*E* mixture of 65:35 (**6**:**7**) showed over short time intervals the consumption of only the *E* isomer (Fig. 1). The relative $V_{max}$ for (*Z*)-**6** and (*E*)-**7** were determined as 0.025% and 52%, respectively, of that observed with PEP under similar conditions. Thus, in contrast to other PEP-utilizing enzymes, which have been shown[11,12] to exhibit either the absolute stereoselectivity or the preferential substrate activity to the (*Z*)-[3-F]PEP, our results reveal that the synthase utilizes both stereoisomers as substrates, but with a high kinetic preference for the (*E*)-[3-F]PEP.

Spectra *a* and *b* in Fig. 2 were recorded for the purified products of the reactions carried out with (*Z*)- and (*E*)-[3-F]PEP, respectively. The signals shown in spectrum *a* were assigned to the tautomeric mixture of (3*S*)-[3-F]KDO8P (**10**, with fluorine atom oriented in axial position) and the signals in spectrum *b* to the (3*R*)-[3-F]KDO8P (**11**, with fluorine atom oriented in equatorial position). The chemical shifts and coupling constants assignments for both products are listed in Table 2. The distribution of tautomers in compound **10** shows a close correspondence to that of KDO8P[10] and appears to be 76 and ~1% to the α- and β-pyranose anomers and 14.5 and 8.5% to the major and minor furanose forms. However, a significant change in the ring and anomeric composition was observed in compound **11**. No indication of β-pyranose form was detected and the observed three signals were assigned to α-pyranose (91%) and major (6%) and minor (3%) furanose forms.

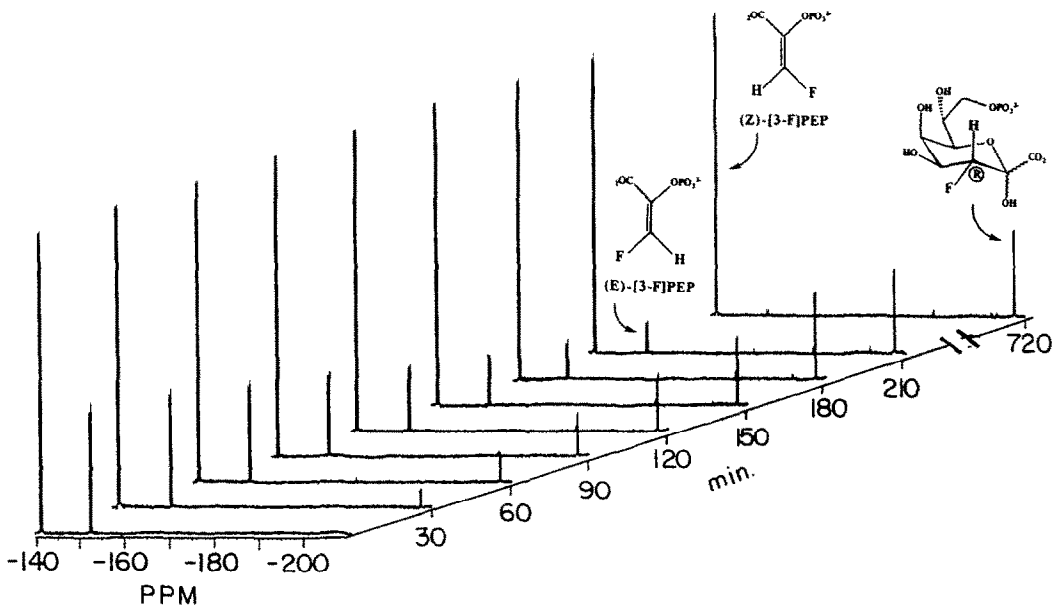



**Figure 1.** A timecourse of $^{19}F$ NMR spectra showing the conversion of (*E*)-[3-F]PEP (7) to (3*R*)-[3-F]KDO8P (**11**) in the presence of Tris-acetate buffer (0.1 M), pH 7.4, A5P (0.11 M), bovine serum albumin (1.5 mg/mL), *Z*-*E* mixture of [3-F]PEP (0.1 M, 6:7 ratio of 65:35, $\delta$ = -141.7 and -153.0 ppm, respectively), KDO8P synthase and 25% $D_2O$ for lock. Spectra were acquired on a Bruker WH-200 operating at 188.313 MHz and are referenced externally to TFA. Insets indicate corresponding structural assignments.

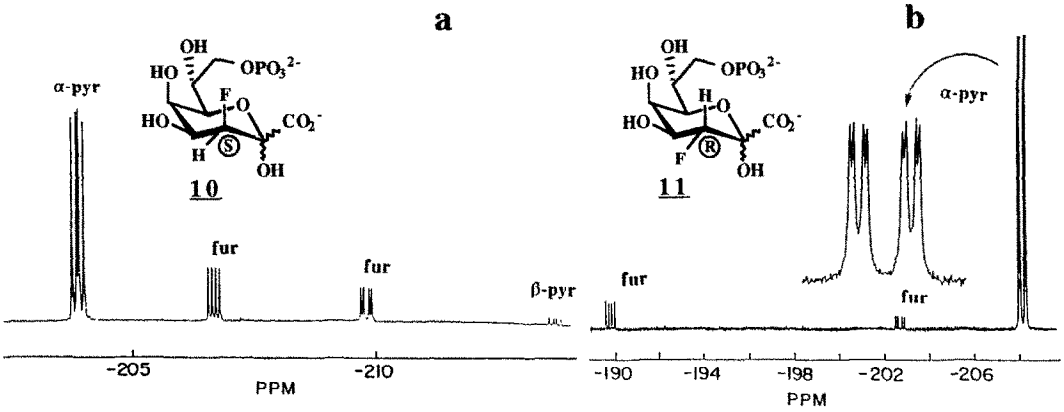



**Figure 2.** $^{19}F$ NMR spectra of (a) (3*S*)-[3-F]KDO8P (**10**); (b) (3*R*)-[3-F]KDO8P (**11**).

**Table 2.** $^{19}F$ NMR Chemical shifts and coupling constants for (3*S*)-[3-F]KDO8P (**10**) and (3*R*)-[3-F]KDO8P (**11**)[a]

| Compound | α-pyranose[e] δ(*J*) | β-pyranose[e] δ(*J*) | major furanose δ(*J*) | minor furanose δ(*J*) |
|---|---|---|---|---|
| **10** | -203.84 (48.8,[c] 34.3) | -214.22 (49.5,[c] 32.9) | -206.66 (53.3,[c] 27.3) | -209.80 (53.3,[c] 17.7) |
| **11** | -208.16 (49.8,[c] 12.8, 3.2[d]) | *b* | -189.74 (51.0,[c] 25.2) | -202.67 (54.5,[c] 19.5) |

[a]Recorded at 188.313 MHz with a Bruker WH 200 instrument, for the potassium salts in $D_2O$, pD 7.2, referenced to TFA at -77.0 ppm. Values of coupling constants are given in parentheses and measured in Hz. [b]Undetected. [c]Geminal $^2J_{F,H}$ coupling. [d] $^4J_{F\text{-}3,H\text{-}5}$. [e]The assignments of the pyranose resonances to α and β are tentative.

The axial orientation of the fluorine in compound **10** was confirmed by the magnitude of the 3-bond coupling constant between fluorine and H-4. This coupling constant in the α- and β-pyranose anomers was 34.3 Hz and 32.9 Hz, respectively, which is only consistent with the *trans*-diaxial ($F_a$, H-$4_a$) orientation, and thus establishes the *S* configuration at C-3 of **10**. However, the same vicinal F-$^1$H coupling constant in the major α-pyranose anomer of compound **11** (Fig. 2b, Table 2) is much smaller ($^3J$ = 12.8 Hz) and is consistent with a gauche ($F_e$, H-$4_a$) orientation of the fluorine and H-4. Such 3-bond coupling constants (12-15 Hz) have been reported[13] for the gauche ($F_e$, $H_a$) arrangements of the fluorine linked to the different carbon atoms of the pyranose ring. Confirmation of the latter assignment was further provided by 4-bond coupling ($^4J$ = 3.2 Hz) between fluorine and H-5 indicating[13] a *cis*-diequatorial orientation of these atoms. The remaining resonances in Fig. 2a,b were tentatively assigned to the major and minor furanose anomers of the corresponding diastereomer. Although these assignments are only tentative, they are not critical to any arguments used in determining the steric course of the enzyme reaction. Finally, the results involving the fluoro analogues of PEP further confirm the *si* face addition of enzyme-bound PEP to the *re* face of the carbonyl of A5P as shown in Scheme 1.

The results of this study define the overall steric course of the KDO8P synthase reaction. Although the exact structure of the reaction intermediate is still unknown,[5] these results pave the way for the solution of the absolute configuration of this intermediate.[14] In addition, the finding of *si* attack at C-3 of PEP may be of interest from the viewpoint of enzyme evolution, since several carboxylases[15], pyruvate kinase,[16] and DAHP synthase,[17] have all been shown to catalyze the similar *si* face addition of PEP.

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